

The Differentiation, Evaluation, and Application of Human Induced Pluripotent Stem Cell Derived Endothelial Cells

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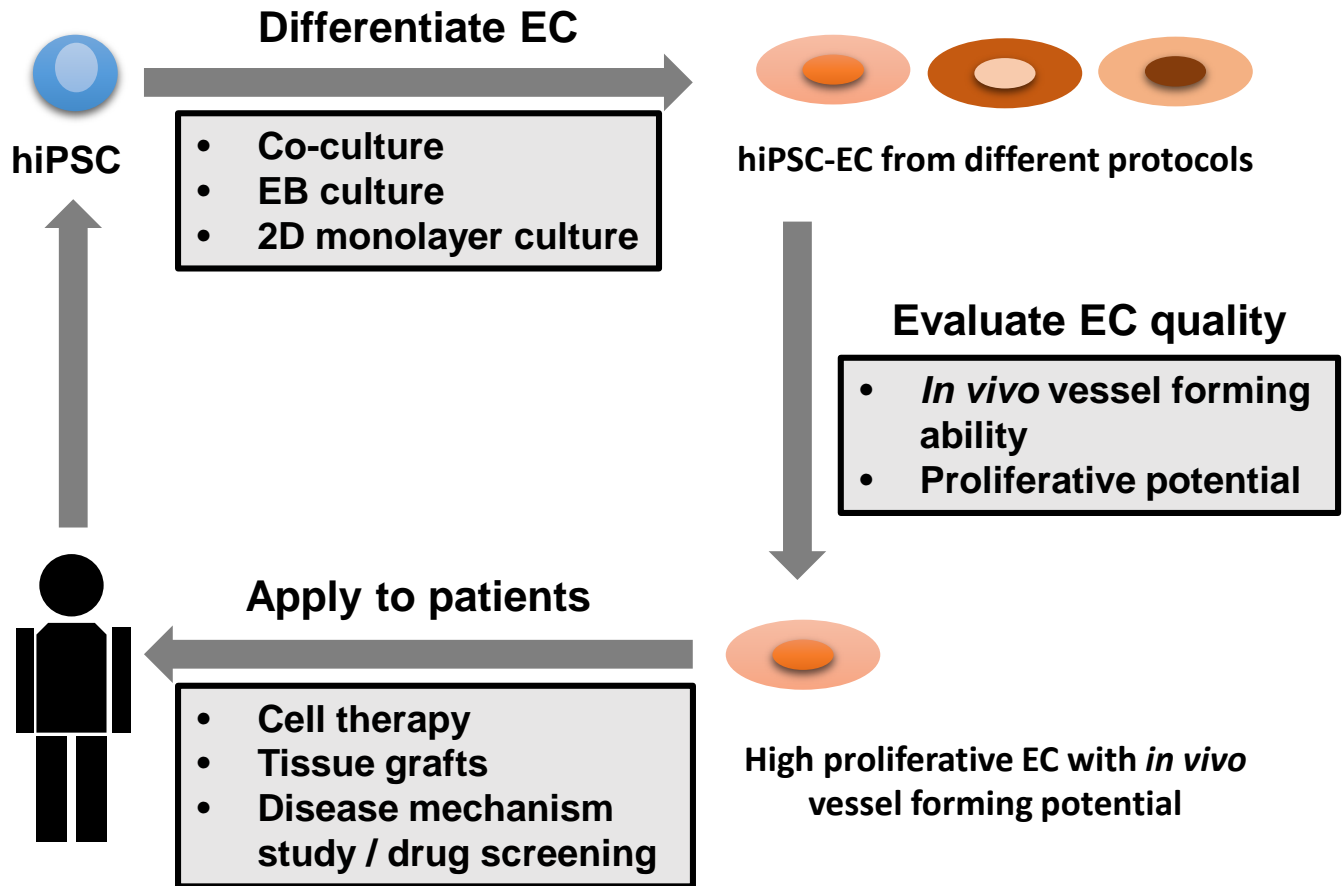
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Abstract

The emergence of induced pluripotent stem cell (iPSC) technology paves the way to generate large numbers of patient-specific endothelial cells (EC) that can be potentially delivered for regenerative medicine in patients with cardiovascular disease. In the last decade, numerous protocols that differentiate EC from iPSC have been developed by many groups. In this review we will discuss several common strategies that have been optimized for human iPSC-EC (hiPSC-EC) differentiation and subsequent studies that have evaluated the potential of hiPSC-EC as a cell therapy or as a tool in disease modeling. In addition, we will emphasize the importance of using *in vivo* vessel forming ability and *in vitro* clonogenic colony forming potential as a gold standard with which to evaluate the quality of hiPSC-EC derived from various protocols.

Key Words: cell therapy ■ differentiation ■ endothelial cell ■ endothelial colony forming cell
■ induced pluripotent stem cell

Graphic abstract



Nonstandard Abbreviations and Acronyms

CRISPR/Cas9	Clustered regularly-interspaced short palindromic repeats-associated protein 9
CVD	Cardiovascular disease
EB	Embryoid body
EC	Endothelial cell
ECFC	Endothelial colony forming cell
ESC	Embryonic stem cell
iPSC	Induced pluripotent stem cell
TALEN	Transcription activator-like effector nucleases

Introduction

Cardiovascular diseases (CVD), which are often triggered by endothelial dysfunction, represent one of the leading causes of mortality in the world. In 2015, over 17 million deaths, which represents 31% of global deaths, were caused by CVD.¹ The treatment of CVD through endothelial replacement is hindered by the lack of methods to isolate sufficient numbers of functionally normal autologous endothelial cells (EC) for transplantation and our limited understanding of how endogenous endothelial cells become dysfunctional during development of various cardiovascular diseases. The innovation of human induced pluripotent stem cells (hiPSC), cells that are reprogrammed from somatic cells to an embryonic stem cell (ESC)-like pluripotent state,^{2, 3} presents an opportunity for us to generate large numbers of patient-specific EC that can be used for transplantation, drug screening, or studies to probe the mechanisms for endothelial dysfunction in certain disease states. Here we will retrospectively catalog and survey the methods developed by various groups to differentiate hiPSC to EC. We will focus on several common topics shared by all the papers, including the strategies for hiPSC-EC differentiation, the importance of functional and phenotypic testing of the hiPSC for quality control, and the potential applications of hiPSC-EC as a cell therapy for human subjects. Articles discussing human ESC derived EC derivation, PSC-EC differentiation from non-human species, and the development of other cell types through EC intermediates, like hemogenic endothelium, will not be covered here and discussions on these topics can be found elsewhere.⁴⁻⁷

Strategies for hiPSC-EC differentiation

Methods to differentiate EC from hiPSC can be divided into 3 general categories: 1) Stromal cell co-culture, 2) Embryoid body (EB) differentiation, and 3) Feeder-free monolayer differentiation (Figure 1).

The methods to differentiate EC from hiPSC by co-culture with stromal cells, usually murine bone marrow derived stromal cell lines like OP9 or M10B2, were mainly used in some of the earliest studies in the field.⁸⁻¹¹ This is an undirected differentiation strategy in which the frequency of differentiated EC is generally low and the EC produced were often mixed with other cell types including hematopoietic cells,⁸ smooth muscle cells,^{9, 10} and inevitably murine stromal cells. Thus this method is not ideal for the generation of a large number of EC for regenerative medicine applications.

EB methods rely on the spontaneous differentiation of aggregated hiPSC in the context of a self-assembled three dimensional (3D) structure. The development of many cell lineages in EB seems to recapitulate the progression of early embryonic development. Thus, the differentiation of EB is also not a fully controlled process and single step long term (10-12 days) EB differentiation method usually gives rise to a low EC yield.¹²⁻²² Many researchers differentiate EB for a shorter period (3-6 days), usually in the presence of added growth factors that promote mesoderm and/or endothelial lineage specification including BMP4, Activin A, bFGF, and VEGF. At this stage, mesoderm and immature endothelial cells are robustly induced. These cells, together with other differentiated cell types from EB,²³⁻³⁴ or enriched by fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) via the use of monoclonal antibodies that identify the expression of mesodermal and/or endothelial cell surface markers like CD309 (KDR),³⁵⁻⁴⁰ CD34,³⁷ and CD31 (PECAM-1),³⁵ are then expanded/enriched in monolayer culture conditions that favors the growth of EC. Some protocols developed using this strategy have differentiated functional EC from human hiPSC that exhibited the ability to form *in vivo* vessels.^{24, 25, 29, 34, 36, 38}

The final general strategy is to culture a monolayer of hiPSC on a matrix coated culture plate and treat them with different molecules or growth factors in a timed fashion to dictate the progressive differentiation from PSC to mesoderm and finally towards the EC lineage. To date,

the protocols that have led to some of the highest EC yields were developed using this general strategy.^{25, 41, 42} Most protocols using this strategy can be divided into a mesoderm differentiation phase and an endothelial differentiation phase. Some protocols will change the culture medium and growth factors between the mesoderm and endothelial stages, but without disturbing the cultured cells,^{25, 41-62} while others purify the mesoderm cells or immature EC through detection of cells expressing KDR,^{54, 63-65} CD34,⁶⁶⁻⁶⁸ MESP1,⁶⁹ or CD31⁶⁷ between these two phases. Once purified the cell populations are further optimized to generate homogenous EC preparations.

Manipulating the signaling pathways that specify mesoderm differentiation from the hiPSC is common to these monolayer differentiation systems. Thus, growth factors like Activin A, BMP4, and bFGF are commonly used. GSK-3 inhibitors like CHIR99021 and BIO are also frequently added to the medium during this phase to promote the canonical Wnt signaling pathway which stimulates the differentiation of the hiPSC derived mesoderm towards the endothelial lineage,^{25, 30, 37, 43, 44, 47, 48, 50, 55, 56, 59, 60, 62, 63, 66, 67, 69} although the effectiveness of GSK-3 inhibition in enhancing EC specification has been questioned.⁶³ Endothelial lineage cells are expanded in the second phase. VEGF is the most common growth factor that is used during this phase. Inhibition of the TGF- β signaling pathway has been reported to be crucial for promoting the endothelial specification from mesoderm cells and the maintenance of EC (to avoid endothelial to mesenchymal transition) in some protocols.³⁰ Thus SB431542, an inhibitor of TGF- β signaling pathway, has often been included in many protocols to maintain and expand EC in the second phase.^{30, 43, 44, 49, 51, 57, 58, 61}

To further improve the differentiation efficiency of EC from hiPSC, a deeper understanding of the development of the EC lineage is required. Researchers are trying to discover new signaling pathways or develop novel culture conditions to improved EC differentiation. Park *et al.*⁶⁸ have reported that inhibition of the MEK/ERK pathway by addition of the inhibitor PD98059 during the mesoderm induction stage increased the production of EC. However, in Lian *et al.*'s study,⁶⁶

addition of the MEK inhibitor PD0325901 decreased the production of CD31⁺CD34⁺ EC. Sahara *et al.*²⁵ reported that inhibition of the Notch signaling pathway during the late phase of EC differentiation, either by transcript knockdown or by addition of the γ -secretase inhibitor DAPT, led to a higher efficiency of EC production. Liang *et al.*,⁵¹ showed that miR-495 could promote the production of EC from hiPSC and improve the angiogenic potential, as well as, the *in vivo* vessel forming ability of hiPSC-EC via interacting with vascular endothelial zinc finger 1. Cochrane and colleagues reported that the RNA binding protein Quaking isoform 5 stabilizes STAT3 and activates the expression of KDR, thus promoting EC differentiation and angiogenesis functions.³⁶ In addition, cyclic adenosine monophosphate (cAMP) has also been added in the EC phase of differentiation to enhance EC enrichment.^{54, 64} Zhang *et al.*⁷⁰ promoted the differentiation of hiPSC into EC in a 3D porous fibrin scaffold and 45% of hiPSC were differentiated to EC. They reasoned that the higher EC differentiation efficiency was due to the 3D surface tension provided by the scaffold.⁷⁰ Recently, Zhang *et al.*⁶⁹ suspended hiPSC derived MESP1⁺ mesodermal cells in a 3D biodegradable gelatin based scaffold and observed more enrichment of the CD31⁺ EC population than from hiPSC-derived mesoderm cells grown in 2D cultures.

Because hiPSC-EC are differentiated *in vitro*, they are not exposed to the fluid flow, pulsatile pressure, or specific tissue microenvironments that impact endogenous EC differentiation *in vivo* and thus, hiPSC-EC display an indeterminate phenotype; neither committed fully to an arterial or a venous fate.²⁹ Rufaihah *et al.*²⁹ discovered that hiPSC-EC derived using the EB differentiation method possess heterogeneous arterial, venous and lymphatic marker expression and they could be directed to differentiate toward the arterial,^{29, 31, 71} lymphatic^{29, 72} or venous²⁹ EC lineages through the manipulation of culture conditions. HiPSC-EC have also been induced in various culture conditions that mimic specific tissue microenvironments to induce tissue specific EC phenotypes. The most extensively studied tissue specific hiPSC-EC phenotype is brain microvascular EC (BEC).^{64, 73-85} Corneal-⁸⁶ and choroidal-⁸⁷ like EC have also been

differentiated from hiPSC. However, at this time none of these *in vitro* induced tissue specific EC has been validated extensively *in vivo*. Thus, whether these cells genuinely represent their *in vivo* counterparts and how long can they keep their tissue-specific phenotype *in vivo* remains unknown.

Quality controls for hiPSC-EC

While EC differentiated from hiPSC through numerous methods exhibit some common traits including, expression of a panel of EC cell surface markers, *in vitro* 2D capillary-like tube forming potential, and ability to uptake low-density lipoprotein (LDL) (Figure 2), these properties alone may be insufficient to assess the function of EC to generate blood vessels *in vivo*. Indeed, many cell types other than EC, like some myeloid lineage hematopoietic cells, can also express EC markers and exhibit tube formation and LDL binding phenotypes *in vitro*^{88, 89}, whereas only bona fide EC form stable, functional blood vessel networks that inosculate with host vasculature upon transplantation.⁸⁸ Thus, *in vivo* vessel forming potential should be a necessary assay of EC derived from hiPSC (Figure 2). One commonly used *in vivo* vessel forming assay is to implant cells within a matrix and deliver the matrix into the subcutaneous space or prepared tissue. In this assay, donor test EC are loaded into a 3D matrix comprised of collagen,^{41, 42} Matrigel,^{16, 19, 24, 25, 29, 34, 36, 38, 40, 68, 90} fibrinogen,⁴⁷ or a degradable hydrogel⁶¹ for transplantation. The matrices can be retrieved from the animals at different time points post-implantation to assess the extent of donor derived vessels, determination of whether or not the donor-derived vessels have inosculated with the host vasculature, and the length of time that the donor cells persist. This method is reproducible (once preliminary studies are completed to optimize the matrix composition, donor cell number, and strain of mice implanted and attention paid to ongoing reagent quality control), straightforward to perform, and thus, is suitable as a standard test to compare the quality of hiPSC-EC derived from different methods of production. Other more invasive methods to assess

endothelial functions of hiPSC-EC have also been tested including, experimental hind limb ischemia,^{22-24, 42, 68, 69} myocardial infarction,^{24, 25, 51} wound healing^{16, 65, 72} and retinopathy^{34, 42} models. Though these models are important for testing the therapeutic potential of hiPSC-EC, these interventional models are influenced by a host of variables including animal strain, sex, age, skill of the technicians, and whether or not the host animals are wild type or carry transgenic modifications. Moreover, in these models, the beneficial effect of the donor cell populations can be conveyed via indirect mechanisms in addition to effects derived from the direct formation of donor derived vessels.⁸⁹ For example, cell types like hematopoietic cells and mesenchymal lineage cells, which are common co-products of many hiPSC-EC differentiation protocols, can also improve blood perfusion by indirect means via secreting angiocrine factors or stabilizing newly formed vessels.^{41, 89, 91} However, these non-EC cell types do not integrate into newly-formed blood vessels.^{89, 92, 93} Thus, in their potential clinical applications, whether they can provide long-term protection to the blood vessels to the hosts, especially those who have EC with compromised proliferative potential,⁴² is questionable. For this reason, whenever these animal disease models are used for testing the function of hiPSC-EC, it is necessary to validate the long-term contribution of donor EC to the host blood vessels. Finally, inclusion of a standard source of primary human EC, such as human umbilical cord venous endothelial cells (HUVEC) or umbilical cord blood endothelial colony forming cells (ECFC) as a control for comparison to the test hiPSC-EC population in the implant assay provides important information as to how similar the test EC are functioning *in vivo*. Recently, *in vitro* 3D vasculogenesis/angiogenesis models that mimic the vessel forming process *in vivo* have been applied to test hiPSC-EC.^{34, 48, 57, 61, 62, 94, 95} In these studies, hiPSC-EC are embedded into 3D matrix made with fibrin⁶¹, collagen,^{34, 48} hyaluronic acid hydrogel,⁵⁷ or poly(ethylene glycol) (PEG) hydrogels^{62, 94, 95} under static conditions^{34, 48, 57, 61, 62, 94, 95} or flow conditions in microfluidic chips⁹⁵ and the hiPSC-EC self-assembled into lumenized blood vessel-like structures.^{34, 48, 57, 61, 62, 94, 95} Transcriptome analyses showed that the gene expression pattern of hiPSC-EC cultured in such conditions are more consistent with that of EC during the *in*

vivo vasculogenesis/angiogenesis process compared to cells cultured *in vitro* under static conditions.^{62, 95} Thus, *in vitro* 3D tube forming assays can be used for pre-screening of functional hiPSC-EC before *in vivo* testing (Figure 2).

Another property of EC that is often neglected in assessing the quality of the EC derived from the various differentiation protocols is a quantitative measure of the proliferative potential of the hiPSC-EC (Figure 2). Evidence from both human and other species has shown that vascular EC are heterogeneous with respect to proliferative potential. Over the past decade, it has been reported that among the EC derived from blood vessels or within the bloodstream, some EC display the potential to clonally propagate *in vitro*, self-renew *in vivo*, and give rise to more EC during vessel growth or regeneration.⁹⁶⁻¹⁰³ Those EC identified by their potential to form endothelial colonies *in vitro*⁹⁶⁻¹⁰² have been called outgrowth EC or endothelial colony forming cells (ECFC).^{98, 104} A single high proliferative potential ECFC can give rise to a colony that contains over 10,000 EC progeny, which may include more progenitors with equal or less proliferative potential,^{97, 98} while more mature EC may not be capable of dividing or giving rise to only a few progeny. Indeed, hiPSC-EC derived from many protocols have displayed restricted proliferative potential and either undergo senescence within 5-8 passages or undergo endothelial to mesenchymal transition (EMT). To date, only a few studies have compared the proliferative potential of different hiPSC-EC fractions and tried to enrich the population of hiPSC-EC with high proliferative potential.^{25, 41, 42} Two studies have compared the colony forming potential of different EC fractions at a single cell level.^{25, 42} Prasain *et al.*⁴² reported that after 12 days of hiPSC differentiation, EC could be discriminated into 2 groups based upon the expression of Neuropilin1 (NRP1), a co-receptor of VEGF and CD31. Only NRP1⁺CD31⁺ hiPSC-EC gave rise to ECFC that contained high proliferative potential when plated in a single cell assay and showed overwhelming superior *in vivo* vessel forming potential after transplantation than the NRP1⁻CD31⁺ population.⁴² Using this culture strategy, hiPSC-EC derived from this protocol can be propagated for up to 18

passages and the hiPSC:EC ratio reached 1:100,000,000 within an 2-3 month period.⁴² In the same year, Sahara *et al.* reported²⁵ that among all hiPSC-EC populations, CD34⁺CD14⁻ EC displayed the highest single cell EC colony forming potential and *in vitro* tube-forming ability. EC derived from this population also exhibited *in vivo* vessel forming potential in Matrigel plug and myocardial infarction models.²⁵ In addition, Samuel *et al.*⁴¹ discovered and reported, that CD34⁺NRP1⁺KDR⁺ EC displayed the highest proliferative potential among all hiPSC-EC tested in a specific protocol. EC from this fraction could be expanded for 15 passages and showed long-term *in vivo* vessel forming potential.⁴¹ However, unlike hiPSC-EC derived from the Prasain⁴² or Sahara²⁵ methods, hiPSC-EC derived from the Samuel *et al.*⁴¹ protocol could not form *in vivo* vessels alone without the co-transplantation of mesenchymal cells.⁴¹ Thus, whether these cell populations represent a similar cell type among all three reports is still elusive. Further efforts to specifically enrich and expand high proliferative ECFC, or an equivalent population, gain a deeper understanding of the mechanisms of EC self-renewal and proliferation, develop new culture strategies to maintain high proliferative ECFC fractions, and to compare and contrast specific ECFC populations derived from hiPSC with EC present in the vasculature of human subjects will be important areas for investigation.

The source of pluripotent cells and hiPSC-EC phenotype

While hiPSC have similar properties and differentiation potential with EC, evidence has emerged that hiPSC possess slightly different gene expression and epigenetic patterns due to their gain or loss of epigenetic memory from their somatic sourced material during the reprogramming process.¹⁰⁵⁻¹⁰⁸ Thus, to safely apply hiPSC-EC for cell therapy or mechanistic studies, it is crucial to understand the differences among EC derived from hESC and those hiPSC derived from various somatic cell origins.

Many studies that differentiated EC from hiPSC line have also tested their protocols with ESC lines and some researchers compared the phenotype and function of EC derived from both sources. With a few exceptions,²⁷ most authors have agreed that hiPSC-EC generally exhibit comparable EC phenotypes and functions with ESC derived EC.^{19, 20, 22, 24, 25, 40, 42-44, 59, 65} Three studies compared the transcriptome between multiple ESC-EC and hiPSC-EC lines differentiated using the EB differentiation method and found that gene expression profiles between ESC-EC and hiPSC-EC showed some variance but, overall were very similar.^{19, 34, 40}

To date, most differentiation protocols have focused on the use of hiPSC derived from fibroblasts, a mature cell type. Some studies compared the EC differentiation potential from hiPSC derived from fibroblasts and other cell sources, include some immature cell populations like cord blood³⁴ or peripheral blood¹⁰⁹ mononuclear cells, bone marrow mesenchymal stem cells (BM-MS)³³ and dental pulp cells,²⁴ or EC like human umbilical cord vein endothelial cells (HUVEC)^{17, 28} and cord blood ECFC.^{43, 44} The conclusions from those studies have provided multiple different interpretations of the importance of the source of cells with respect to derivation of hiPSC-EC. While some reports suggest immature cells or EC derived iPSC have a higher tendency to differentiate to EC when compared with fibroblast derived iPSC.^{28, 33, 34} others showed similar or highly variable differentiation efficiency between these two groups.^{24, 43, 44} Obviously, more studies are needed to answer this question.

The applications of hiPSC-EC

EC derived from patient-specific hiPSC can provide a nearly limitless source of autologous cells for various applications against CVD. The most direct application of hiPSC-EC would be to provide these cells as a therapy to replace injured or dysfunctional EC present in the systemic vasculature. Indeed, in some cases, such as patients suffering critical limb ischemia (CLI), evidence has been presented that there is loss of the microvasculature and within isolated blood

vascular EC from these patients, high proliferative ECFC are completely absent.⁴² It has been shown that hiPSC-EC from various protocols form functional blood vessels upon transplantation in a 3D gel matrix^{16, 19, 24, 25, 29, 34, 36, 38, 40-42, 47, 61, 68, 90} or into zebrafish embryos,^{43, 44} suggesting that hiPSC-EC display potential to treat CVD by directly forming vessels and improving blood perfusion. Some other cell types, like mesenchymal and hematopoietic lineage cells, can also promote blood vessel growth and improve blood perfusion through indirect mechanisms in these animal models^{41, 88, 90}. However, these non-EC cell types generally do not integrate into the newly formed blood vessels as long-term components,^{89, 92, 93} thus as a potential cell therapy, repetitive delivery of these cells might be necessary. Likewise, though some recent evidence show that extracellular vesicles, like microvesicles and exosomes from different cell source could also promote angiogenesis,^{110, 111} their short-half life and low encytosis rate are potential hurdles for their clinical applications.¹¹⁰ In addition, all in-direct pro-angiogenic effects from these non-EC cell types or extracellular vesicles rely on the communication between these cells/vesicles and local EC in host vessels. Thus for those patients who suffer from diminished EC proliferative potential, like CLI patients,⁴² the therapeutic benefits of non-EC agents are unlikely to be effective. In contrast, hiPSC-EC have the potential to form stabilized functional vessels and thus provide long-lasting support to the patients' blood vessel system. Indeed, although to date, no clinical trail using hiPSC-EC on human CVD patients has been performed, the therapeutic potential of hiPSC-EC has already been tested in many pre-clinical animal studies including hind limb ischemia,^{22-24, 42, 68, 69} myocardial infarction,^{24, 25, 51} retinopathy,^{34, 42} carotid artery injury²⁶ and dermal wound healing models.^{16, 65, 72} In those studies, *in situ* administration of hiPSC-EC improved the recovery of model animals after injury and most reports^{16, 22-25, 34, 42, 51, 68, 69, 72} highlighted significant levels of integration of donor cells into the host vasculature after transplantation. Thus, patient subjects suffering from peripheral artery disease, delayed wound healing, acute and chronic heart failure, stroke, and certain forms of retinopathy that are associated with endothelial dysfunction and vascular dropout are prospective target groups to benefit from this form of cell therapy (Figure 3).

Other than direct administration of EC suspensions, hiPSC-EC can also be mixed with other cell types in a 3D scaffold to assemble transplantable tissue grafts. Donor EC formed blood vessels can promote the connection of a graft into the host vascular system thus improving the survival of grafts after transplantation. To date, liver,⁴⁶ cardiac^{10, 45, 53, 112} and skin grafts⁵⁰ have been developed *in vitro* and transplanted into injured animals. In all these cases, the engrafted animals showed improved recovery and inosculated donor vessels.^{10, 45, 46, 50, 53, 112} In addition, Lu *et al.*³⁹ have reported that decellularized murine heart scaffolds could be recellularized with hiPSC derived multipotent cardiovascular progenitors to engineer heart tissue with contractile function (Figure 3). Other than those tissue grafts, miniaturized organoids that mimics the structure and function of *in vivo* organs including blood vessel,¹¹³ kidney,^{114, 115} liver,¹¹⁶ cornea,¹¹⁷ and retina,¹¹⁸ in 3D culture have also been constructed using hiPSC-EC and other cell types. These organoids can be potentially used for drug screening, disease study, or cell therapy as units of an artificial tissue graft.

Patient-specific hiPSC-EC can also be potentially used for studying disease mechanisms or drug screening to find new molecules to modulate pathways contributing to endothelial dysfunction in a patient and disease specific fashion. Compared to animal models, models using patient derived hiPSC-EC may provide more human-relevant information on disease mechanisms or drug responses (Figure 3). Recently, the pool of CVD patient specific hiPSC lines is expanding rapidly. Disease specific hiPSC-EC have been generated and studied to model BMPR2 mutation caused pulmonary arterial hypertension,¹¹⁹⁻¹²¹ Moyamoya disease,^{56, 58} fibrodysplasia ossificans progressiva,³⁵ Huntington's Disease,⁷⁸ Kawasaki disease,¹⁰⁹ type I diabetes,^{41, 57, 122} atrial or ventricular septal defects, pulmonary valve stenosis, cardiomyopathy,¹²³ calcified aortic valve disease (CAVD),¹²⁴ and Hemophilia A (HA).¹¹ We have summarized above-mentioned disease studies that have characterized disease patient hiPSC derived EC in Table 1. For more information about patients derived hiPSC lines, please check International Stem Cell Registry

(<https://www.umassmed.edu/iscr>) or CIRM human pluripotent stem cell registry (<https://catalog.coriell.org/1/CIRM>).

Along with the development of advanced gene editing tools such as transcription activator-like effector nucleases (TALEN) and CRISPR/Cas9, more and more new strategies using targeted gene correction to treat or study cardiovascular related diseases have been developed. To understand the effect of BMPR2 mutations in EC, Gu *et al.*¹²¹ compared EC differentiated from familial pulmonary arterial hypertension (FPAH) patient derived hiPSC, normal control patient derived hiPSC, unaffected BMPR2 mutation carrier patient derived hiPSC, and CRISPR/Cas9 rescued FPAH patient derived hiPSC. EC differentiated from FPAH patient derived hiPSC with BMPR2 mutations possessed impaired EC functions and survival, while unaffected carrier patient hiPSC or BMPR2 rescued FPAH patient hiPSC derived EC were relatively normal, suggesting additional BMPR2 modifiers play a role for the protection of unaffected carriers from FPAH phenotype.¹²¹ Theodoris *et al.*¹²⁴ compared gene expression and the epigenetic landscape between EC derived from CAVD patient iPSC with *NOTCH 1* mutation and TALEN rescued isogenic iPSC and discovered *NOTCH1* haploinsufficiency caused epigenetic changes that lead to the up-regulation of osteogenesis gene expression in CAVD patient EC.¹²⁴ Yang *et al.*¹²⁵ surveyed the genome of patients with BAV and identified *GATA4* as a target of mutation in these patients.¹²⁵ EC differentiated from CRISPR/Cas9 induced *GATA4* targeted mutation hiPSC were then used to confirm this mutation is associated with impaired endothelial to mesenchymal transition.¹²⁵ Wu *et al.*¹¹ have recently generated hiPSC lines from a patient with HA caused by a factor eight (FVIII) gene intron 22 inversion and used TALEN to correct this specific mutation. Corrected HA patient specific hiPSC-EC restored FVIII transcription and secretion.¹¹

As a platform for high-throughput drug screening and toxicity testing, hiPSC-EC can potentially be used to generate personalized therapeutic strategies. Recently, Vazao *et al.* developed a method to screen drugs that could potentially affect embryonic vasculature

development and identified two drugs, fluphenazine and pyrrolopyrimidine, that could decrease EC viability and disrupted EC network formation.¹²⁶ Sharma *et al.* generated hiPSC-EC as well as cardiomyocytes and fibroblasts from 11 healthy patients and 2 cancer patient to test the cardiotoxicity effect of 21 FDA approved anti-cancer tyrosine kinase inhibitors and developed a “cardiac safety index” for those drugs.¹²⁷ To better mimic *in vivo* environments in which EC constantly undergo shear stress from blood flow and often interact with other local cell types, new microfluidic 3-D platforms like “organ-on-a chip” have been developed and tested for drug screening on various *in vitro* organ mimics.^{128, 129} Recently, Wang *et al.* developed an *in vitro* BBB microfluidic model using hiPSC-EC and validated the potential of this approach for screening drugs that affect EC permeability.¹³⁰ As more sophisticated *in vitro* vascularized organ models are developed, it can be expected that pre-clinical drug development process for CVD can be greatly shortened.

Challenges for the applications of hiPSC-EC as cell therapy

IPSC technology has only been developing as a discipline for a decade. Though hiPSC has been extensively applied for *in vitro* mechanistic studies, their application as cell therapy still face numerous practical problems, like low reprogramming efficiency,¹³¹ genetic instability,¹³²⁻¹³⁴ and potential of teratoma formation from undifferentiated iPSC.¹³⁵ To date, only one clinical trial has been completed in human subjects, using hiPSC derived retinal pigment epithelial cells,¹³⁶ and the safety and effectiveness of hiPSC therapy are still under evaluation. In general, application of hiPSC derived cells Cell therapy for CVD will require a large number of EC to replace the dysfunctional EC present in specific tissues and organs. However, most hiPSC-EC differentiation protocols that have been developed so far generate low EC yields and only produce mature EC with limited proliferative potential. Scaling-up the starting hiPSC cell number can partially solve

this problem but may not be practical or cost-efficient. Recently several groups have started to focus on differentiating hiPSC into EC precursors that display high proliferative potential.^{25, 41, 42} It can be predicted that along with a better understanding of the mechanisms of EC lineage specification and proliferation, more efficient proliferative EC differentiation strategies should be developed to get clinical relevant numbers of hiPSC-EC for cell therapies.

The second limitation for current hiPSC-EC studies is the lack of a commonly-accepted standard to evaluate the quality of hiPSC-EC derived from various protocols to assess their potential for clinical use. While the route of delivery and the dose of cells to be used need to be determined case-by-case for each therapy, a basic set of quality control requirements for a hiPSC-EC therapeutic should be discussed and evidence based. However, many *in vitro* assays that are frequently used to define the quality of the hiPSC-EC, like CD31 expression, LDL binding, or 2-D Matrigel network formation, cannot definitively predict the ability of the EC to form vessels *in vivo*.^{88, 89} Thus, to evaluate the quality of a hiPSC-EC product, some form of testing to validate *in vivo* vessel formation in animal models is recommended.

The next challenge for the practical utilization of hiPSC-EC is developing differentiation protocols that mimic the heterogeneous nature and tissue specificity of vascular EC. It is well known that arterial, venous, lymphatic and capillary EC show different patterns of gene expression and function.¹³⁷⁻¹³⁹ Additionally, EC from each organ vascular bed share some common features, but also show unique gene expression signatures.¹⁴⁰ While most hiPSC-EC are not fully committed to any specific EC phenotype, several studies have attempted to induce hiPSC-EC to express tissue specific functions^{64, 73-87} or arterial/venous/lymphatic EC phenotypes.^{29, 31, 71, 72} For example, *in vitro* cultured ECFC, which mostly form capillary EC after transplantation, also possess the ability to differentiate into arterial EC *in vivo* when constitutively activated NOTCH expressing stromal cells are co-implanted.¹⁴¹ The local tissue microenvironment has been reported to “educate” murine ESC derived EC to adapt the gene expression pattern of tissue

specific EC upon transplantation.¹⁴⁰ Thus, some EC possess the capacity to adapt to local environmental cues *in vivo* and further work to develop similar responsive hiPSC-EC are underway.

Finally, although autologous patient derived hiPSC-EC are promising for the treatment of chronic CVD, the process of hiPSC induction and EC differentiation can take several months to complete, which makes their potential for treating acute injuries unlikely. Some investigators are directly reprogramming somatic cells into EC without a pluripotent intermediate.¹⁴²⁻¹⁴⁴ At present it is unclear if the reprogrammed EC possess high proliferative potential. As an alternative strategy, some investigators are proposing to use banked allogeneic haplotype-matched hiPSC to produce cell products to diminish the potential for immunologic detection and elimination.¹⁴⁵ Other strategies include development of hiPSC lines that are genome edited to make universal donor cells that lack major histocompatibility antigens I and II that should be immunologically well tolerated by the host unmatched patients.¹⁴⁶⁻¹⁴⁸ These strategies will be helpful in overcoming some of the practical issues for production of banks of hiPSC derived EC for cell therapy.

Conclusions

In the past decade, numerous methods to differentiate EC from hiPSC, have been developed and the potential of applying hiPSC-EC in regenerative medicine approaches have been tested in various animal models of human disease. In addition, many patient-specific hiPSC lines have been generated and EC differentiated from these lines have been used for disease mechanism exploration and discovery. The accumulating knowledge on hiPSC-EC differentiation, functional evaluation and potential application will benefit the development of new strategies against CVD.

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Highlights

- Numerous protocols have been developed to differentiate hiPSC into EC using stromal cell co-culture, embryoid body (3D) culture, or 2-D monolayer culture strategies.
- The *in vivo* blood vessel forming potential and *in vitro* clonal proliferative potential should be assayed as a quality control measure before using hiPSC-EC for cell therapy.
- HiPSC-EC can be employed in patients with cardiovascular disease through direct cell administration, implantation of engineered tissue grafts, or as models for studying the disease mechanisms causing EC dysfunction, or for drug screening.

Table Legend

Table 1. List of publications using patient derived hiPSC-EC for disease study

Figure Legends

Figure 1. General strategies for hiPSC-EC differentiation.

Figure 2. General methods to evaluate the quality of hiPSC-EC.

Figure 3. Potential applications of hiPSC-EC.

Table 1. List of publications using patient derived hiPSC-EC for disease study

Disease	Mutation	EC Phenotype	Year	Authors	Reference
Moyamoya disease	RNF213 R4810K polymorphism	-In vitro tube formation↓ -Securin expression↓	2013	Hitomi <i>et al.</i>	56
Pulmonary arterial hypertension (PAH)	BMPR2	-Differentiation↓ -LDL uptake↓ -In vitro tube formation↓ -Wnt signaling↑	2014	West <i>et al.</i>	119
Type I diabetes (T1D)	-	-Differentiation no effect	2015	Chan <i>et al.</i>	57
Calcified aortic valve disease (CAVD)	NOTCH1 haploinsufficiency	-Epigenetic architecture change - pro-osteogenic genes↑ -Inflammatory genes↑	2015	Theodoris <i>et al.</i>	124
Hemophilia A (HA)	FVIII intron 22 inversion	-FVIII secretion↓ -FVIII activity↓	2016	Wu <i>et al.</i>	11
Fibrodysplasia ossificans progressiva (FOP)	ACVR1 R206H	-BMP4 independent differentiation -Fibroblast related gene expression↑ -BMP4 induced SMAD1/5/8 phosphorylation↑	2016	Barruet <i>et al.</i>	35
Moyamoya disease	RNF213 R4810K	-In vitro tube formation↓ -Proliferation no effect -Integrin $\beta 3$ expression↓	2016	Hamauchi <i>et al.</i>	58
Intravenous immunoglobulin (IVIG) resistant Kawasaki disease	-	-CXCL12 expression↑ -Interleukin-6 pathway↑	2016	Ikeda <i>et al.</i>	109
Atrial or ventricular septal defects, pulmonary valve stenosis, cardiomyopathy	GATA4 G29S	-EC related gene expression↑	2016	Ang <i>et al.</i>	121
Huntington's Disease (HD)	CAG repeat expansion in Huntingtin (HTT)	-In vitro tube formation↑ -Barrier function↓ - WNT/ β -Catenin pathway↑	2017	Lim <i>et al.</i>	78
Pulmonary arterial hypertension (PAH)	-	-In vitro tube formation↓ -Adhesion↓ -Migration↓ -Survival↓ -BMPR2 expression↓ -Kisspeptin 1 expression↑	2017	Sa. <i>et al.</i>	120
Familial pulmonary arterial hypertension (FPAH)	BMPR2	-In vitro tube formation↓ -Adhesion↓ -Migration↓ -Survival↓	2017	Gu <i>et al.</i>	121

Figure 1

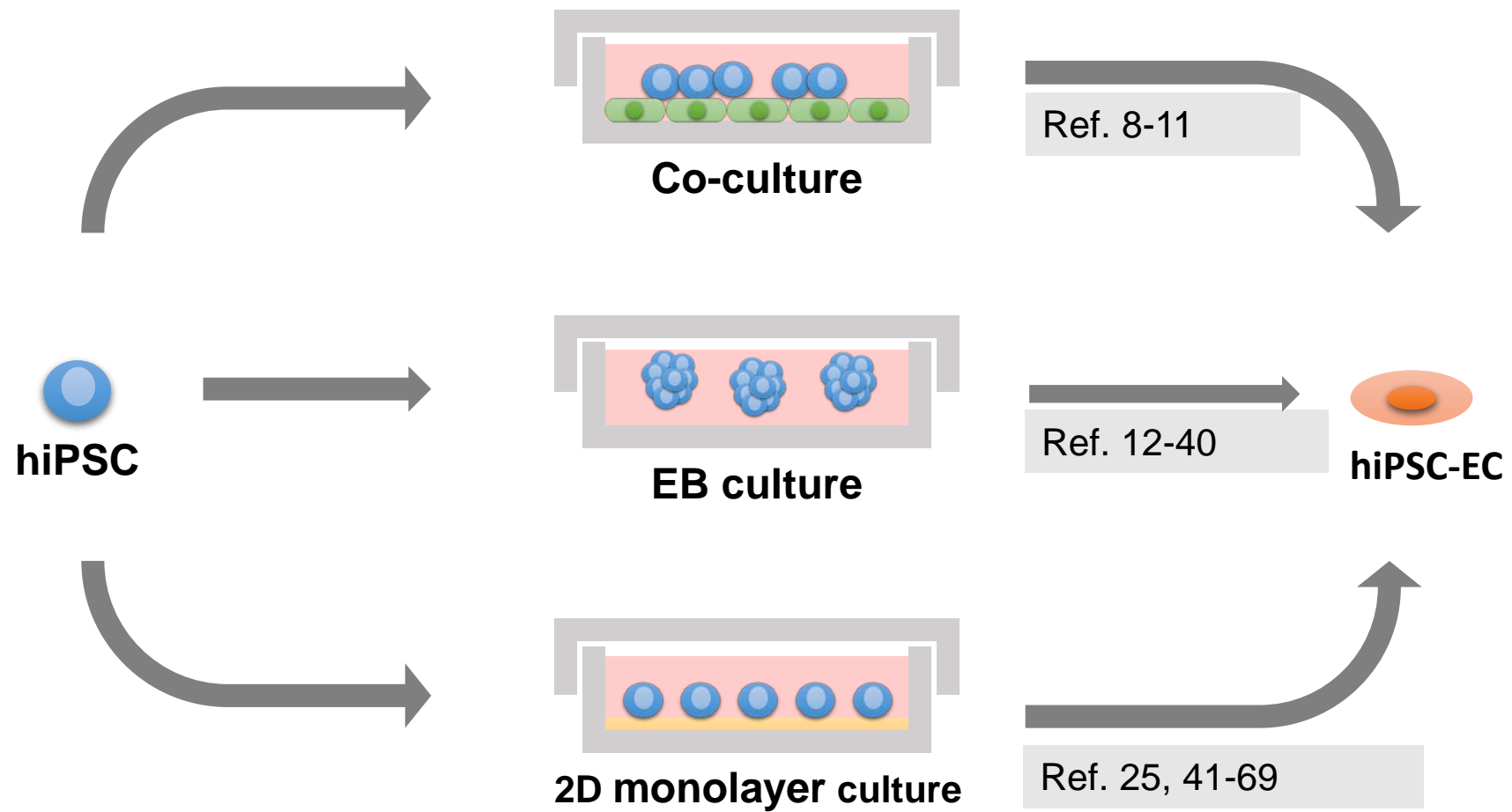


Figure 2

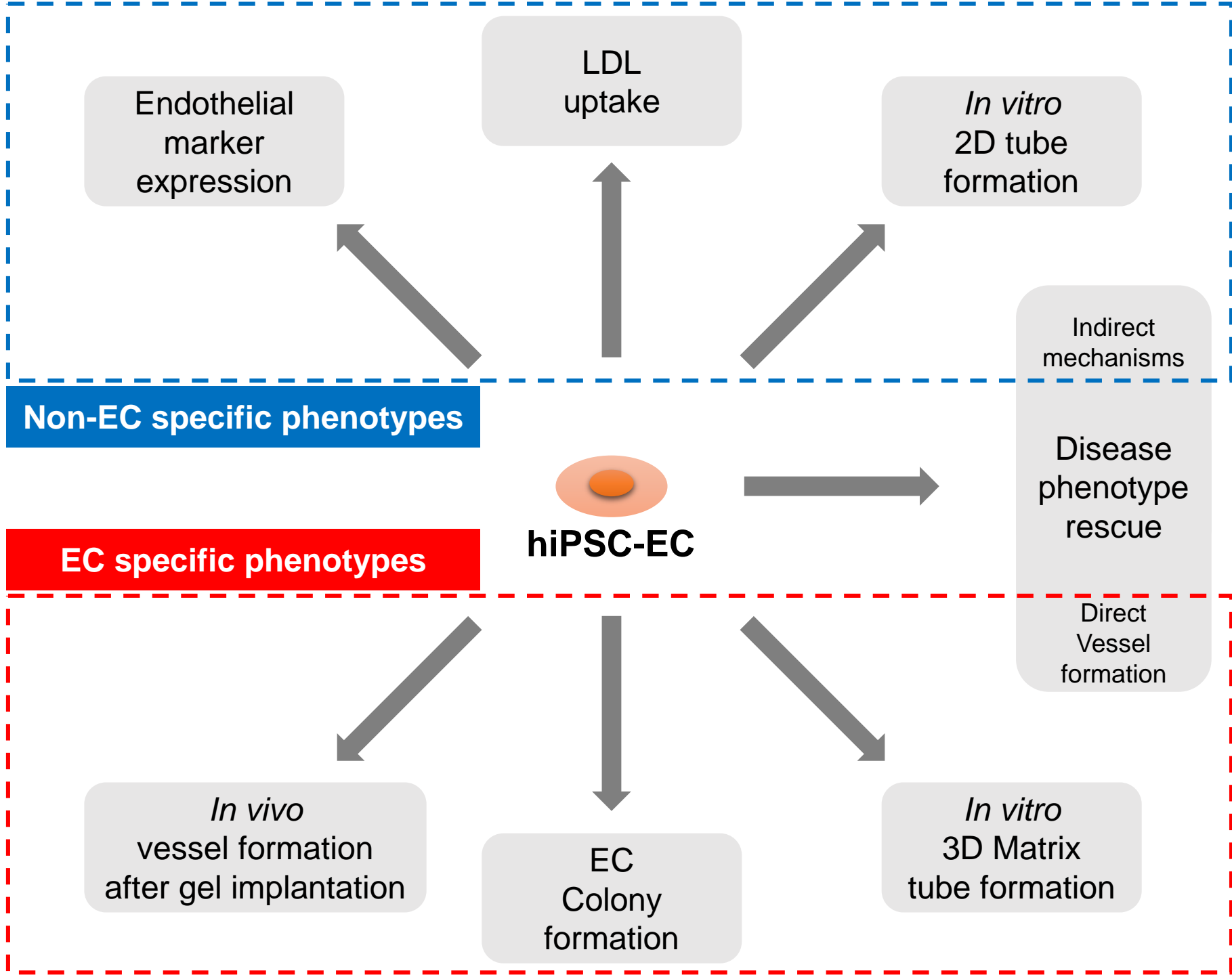


Figure 3

